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13. ABSTRACT (Maximum 200 Words)

The goal of my traineeship is to employ genetic approaches to investigate PTEN signaling in prostate cancer. My first task was to construct expression vectors for a series of tumor-derived PTEN mutations and test these for the ability to perform a diverse set of PTEN functions. This mutant analysis is 75% complete and will be submitted for publication this year. My second task was to set-up and perform a large-scale RNAi screen to identify novel components involved in PTEN signaling. Setting-up this screening system has proven to be very difficult. After trying many approaches it appears that we have finally identified a tractable screening system. Initially, we tried to set-up to do a luciferase reporter screen, but the dynamic range was not suitable for screening. We switched to doing a suppressor screen using inducible PTEN. My second year of funding will primarily focus on performing this screen. While setting up the PTEN RNAi screen we found that a TRAIL luciferase reporter can be used to monitor endogenous Pi3K pathway activity from the insulin receptor to the transcriptional outputs; this work now comprises a full manuscript which will be submitted for publication.

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Introduction

Acquiring inactivating mutations in tumor suppressor genes facilitates cancer progression. *PTEN* encodes a tumor suppressor that is lost in 29% of all prostate tumors and 60% of advanced prostate tumors (4,5,12). The loss of *PTEN* diminishes the effectiveness of chemotherapeutic treatments for cancer (8). By studying the mechanisms involved in *PTEN* signal transduction, we hope to learn ways to better treat prostate cancer. Toward this goal, we proposed to investigate the ability of PTEN mutants to perform known PTEN functions as well as perform a screen to identify novel components involved in PTEN signaling. Our ultimate goal is to identify essential PTEN targets that suppress tumor progression, to study the regulation of these targets and to devise new ways to activate these targets in the absence of PTEN. The ability of PTEN to inhibit tumor progression is well characterized in murine models and tissue culture systems (1-3,6,7,9-12). In mice the homozygous loss of PTEN is lethal whereas the heterozygous deletion leads to cancer including prostate cancer (1,3,9,11). Tissue culture studies have established PTEN as a major regulator of proliferation, cell migration, cell polarity and apoptosis. PTEN is a dual specificity phosphatase that removes phosphates from protein targets and arguably more pertinently to cancer can dephosphorylate lipid second messengers such as phosphatidylinositol 3,4,5-triphosphate [PtdIns (3,4,5) P_3] at the D3 position. The diminishment of PtdIns (3,4,5) P_3 imposed by PTEN inhibits numerous Pleckstrin homology domain containing proteins such as Akt by blocking their membrane recruitment and subsequent activation. The inhibition Akt acts as a cellular break on proliferation and cell cycle progression while also affecting apoptosis. The work described in this report closely follows the work detailed in my Statement of Work. In brief, I outlined two tasks in the Statement of Work. The first task was to test the hypothesis that certain PTEN functions may be separable. To test this hypothesis, I constructed expression vectors for 23 different PTEN mutants; see Table 1. These mutants were tested for the ability to perform its known functions. I found that subsets of mutants that appear to perform distinct subsets of its functions. We are investigating the mechanisms by which these mutants perform distinct PTEN functions. Task two entails screening for novel components involved in PTEN signaling. First, we developed a reporter system for PTEN signaling. This reporter system is not suitable for large scale screen, but still is very useful for studying PTEN signaling. An inducible PTEN containing cell line is our current system for performing a large scale screen to identify novel components in PTEN signaling.

Body

Task 1:

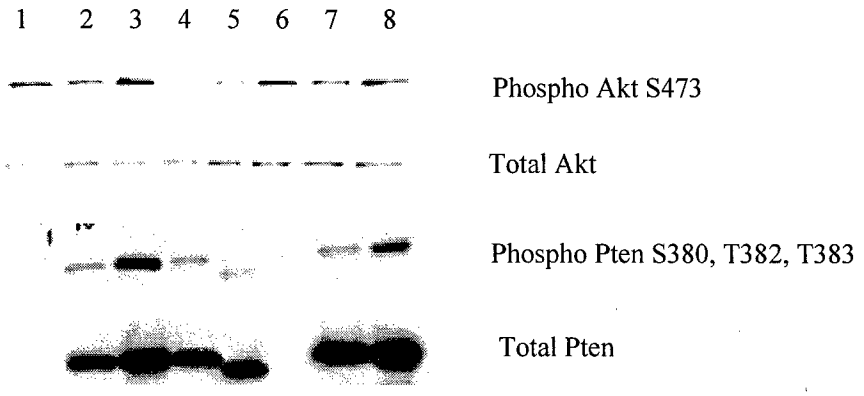
Almost all of the *PTEN* mutants found in tumors greatly diminish phosphatase activity and/or protein stability. To date, a small, C-terminal region of PTEN has been mapped as being important for directing function during cell migration as well as regulating overall PTEN activity and stability. Although initial efforts to investigate PTEN signaling mechanisms have proven informative, the mechanisms responsible for activating, down regulating and directing PTEN activity are still largely unspecified. The ability of PTEN to dephosphorylate PIP3 and thereby negatively regulate Pleckstrin homology (PH) domain-containing proteins such as AKT is well documented. This inhibition leads to numerous signaling events that affect proliferation, apoptosis, cell size and motility. In order to specifically regulate these activities, we suspect that PTEN regulates multiple, independent PIP3 pools that are linked to different receptor signaling complexes. As one means to test this hypothesis, we have constructed expression vectors for 23 mostly tumor derived PTEN mutants. Most of these mutants were previously shown *in vitro* to retain catalytic activity (13).

Table 1. PTEN MUTANTS

Mutation	catalytic activity
1. S10N	+
2. R15S	+/-
3. R15I	+/-
4. G20E	+
5. L42R	+
6. C71S	
7. C124S	-
8. G129E	+/-
9. M134L	+/-
10. L186V	+
11. S227F	+
12. K289E	+
13. D331G	+/-
14. K342X	+/-
15. F347L	+/-
16. Y369G	+
17. V119L	
18. V158L	
19. S287A	
20. S287E	
21. S370A	
22. S370E	
23. C2 loop deletion	

We are investigating the ability of these mutants to affect colony formation, AKT signaling, PTEN phosphorylation, IRS2 induction, ERK phosphorylation and S6 phosphorylation. We have tested the ability of these mutants to function in many different cell lines including 293 cells, U87MG's, BT549's DU145's, LNCaP's and PC3's. Of note, these experiments include work with the three most commonly available prostate cancer cell lines (DU145, LNCaP and PC3) in order to ensure that findings are relevant in prostate cancer. By performing this mutant analysis, we have identified mutants with intermediate phenotypes and are testing whether these mutants only perform subsets of PTEN functions. We strongly suspect that this is the case, but would like to more rigorously test this. Mapping distinct functions to particular regions within PTEN will speak toward mechanisms employed to regulate and direct PTEN action. In addition, missing functions in these tumor derived mutants are most likely to be critical in mediating tumor suppression.

The analysis of PTEN mutants has involved the use of two vector systems IRESpuro and pCEP4. The IRES vector was used in our initial experiments in which we performed a western blotting analysis and reporter assays to assess the ability of these mutants to regulate AKT, ERK, IRS2, PTEN and a FOXO-regulated luciferase reporter TRAIL. We switched vector systems to pCEP4 in order to perform colony formation assays, because the IRES vector was not tractable for this application. Pictured below is a typical western blot experiment in which we tested for the ability of the mutants to regulate the phosphorylation of AKT on serine 473 as well as regulate PTEN phosphorylation on S380, T382 and T383. Western blotting experiments are being done with lysates from PC3's and U87MG's. Our western blotting results from these cells lines are in most instances identical. One can see that wild-type PTEN diminishes Phospho-AKT S473 whereas some of the mutants fail to regulate AKT, Figure 1.



Key:

1=IRES; 2=PTEN; 3=C124S; 4=K289E; 5=C2loop deletion; 6=S287A; 7=S287E; 8=C124A

Figure 1. PTEN mutants vary in the ability to regulate AKT and show differential levels of phosphorylation on the C-terminal residues (S380, T382, T383). PTEN deficient U87-MG cells were transfected with the vector alone (IRES), PTEN, or the indicated PTEN mutant using the CaPO4 method of transfection. Lysates from these samples were used for western blotting analysis. The level of AKT phosphorylation was assessed by probing western blots with antibody that recognizes AKT when phosphorylated on residue serine 473. As a control, we also probed for total AKT. The increase in phosphor-AKT on serine 473 was normalized by the total AKT to determine whether it was increased or not in comparison to control samples. We also performed tubulin loading controls for these experiments (not shown). In addition, we probed blots with a phospho-specific antibody for PTEN as well as antibody to total PTEN. We assessed the level of phospho-PTEN by normalizing levels to total PTEN.

We also tested whether the mutants were able to induce the expression of a FOXO transcription factor-regulated TRAIL luciferase reporter, Figure 2. We found that mutants that were able to negatively regulate AKT were also able to induce TRAIL reporter expression in every instance tested. This is consistent with what is already known about FOXO regulation by AKT (2). Interestingly, even though most of the mutants were reported previously to retain catalytic activity *in vitro*, they failed to regulate AKT and induce the TRAIL reporter in our assays. Therefore, it's clear that most of the mutants are endogenously defective in performing known tumor suppressive activities.

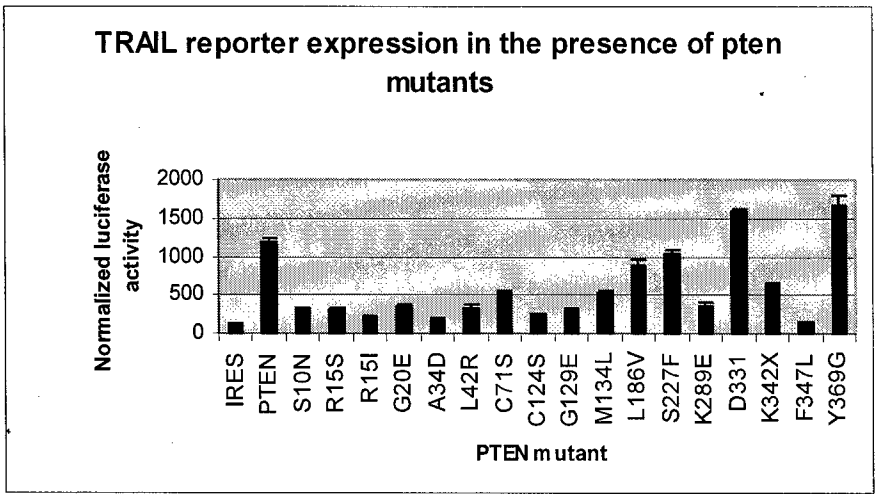


Figure 2. PTEN mutants differentially regulate the TRAIL reporter. 293 cells were transfected with 1 microgram of the TRAIL reporter with either 500 nanograms of the vector alone or PTEN vector using Lipofectamine 2000 (Invitrogen). In addition to these components all transfections included 100 nanograms on the TK-Renilla luciferase reporter to be used as a control for cell number and transfection efficiency. Luciferase assays were performed using the Dual Luciferase Assay Kit from Promega. Reporter activities were normalized with the TK-Renilla luciferase control obtained from Promega. All assays were performed in triplicate. The depicted data is represented as ratios of normalized reporter activity with exogenous PTEN divided by the normalized reporter expression with the vector alone. These data show that wild-type PTEN induces the TRAIL reporter whereas PTEN mutants vary in the ability to induce this reporter.

Colony formation assays revealed that some of the mutants that were able to induce TRAIL expression and regulate AKT were still defective for colony formation inhibition; the L186V mutant falls into this category (Figure 3). These mutants appeared to be defective in regulating phospho-ERK by western blot (not shown); this is being more rigorously tested. Also, the L42R mutant lacked the ability to regulate AKT and induce TRAIL, but showed an intermediate degree of colony suppression. This mutant appears to retain some other PTEN function that inhibits colony formation.

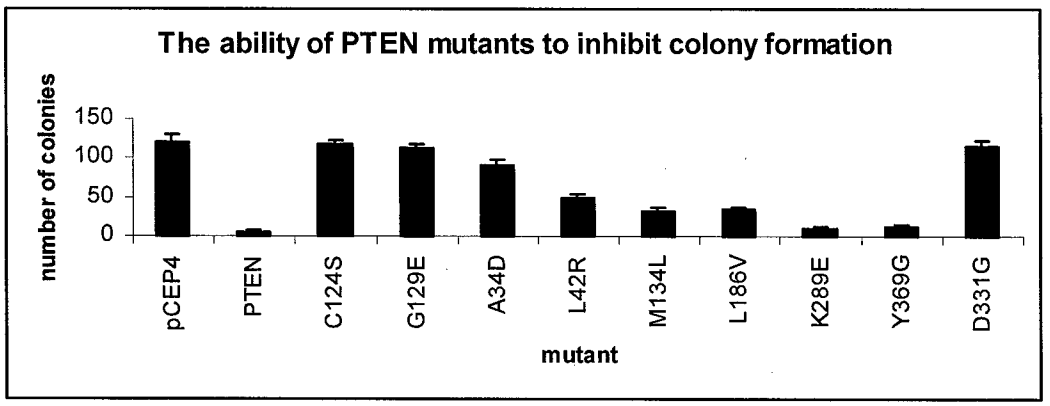


Figure 3. The PTEN mutants differentially affect colony formation. PTEN-deficient BT549 cells were transfected with the indicated vector and the ability to from colonies was assessed. All samples were done in triplicate. These results show that the addition of exogenous PTEN greatly diminishes colony formation whereas many of the mutants fail to do inhibit colony formation.

These results suggest that groups of mutants are defective for subsets of PTEN functions. One possibility is that one group is able to regulate growth whereas the other is able to regulate apoptosis. These

possibilities are currently under investigation. We expect to complete western blotting experiments, colony formation assays as well as follow-up experiments for the mutant analysis by the end of this summer and will then submit this project for publication.

Task 2:

The second major aim for my traineeship is to perform a large-scale RNAi screen to identify novel components involved in PTEN signaling. Initially, we set out to identify a luciferase reporter that could be used as a barometer of PTEN signaling. First, we compared the ability of FOXO regulated luciferase reporters to be induced by PTEN; the TRAIL reporter was induced by ten fold.

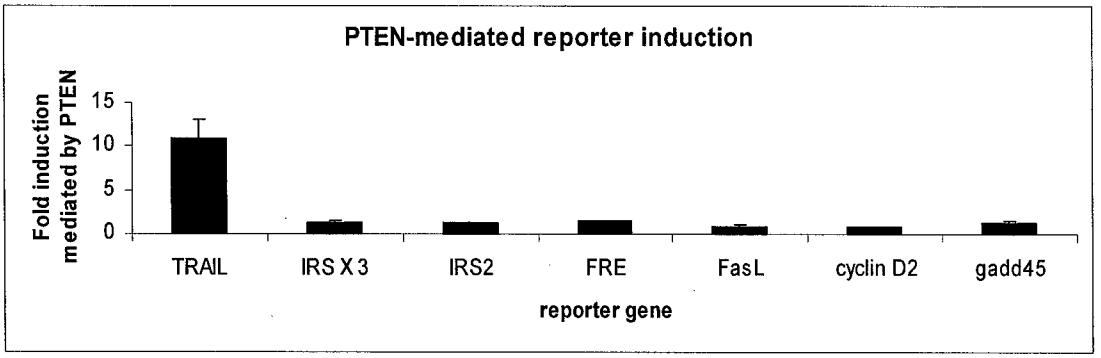


Figure 4. The TRAIL reporter is induced by PTEN. 293 cells were transfected with 1 microgram of each indicated reporter with either 500 nanograms of the vector alone or PTEN vector using Lipofectamine 2000 (Invitrogen). In addition to these components all transfections included 100 nanograms on the TK-Renilla luciferase reporter to be used as a control for cell number and transfection efficiency. Luciferase assays were performed using the Dual Luciferase Assay Kit from Promega. Reporter activities were normalized with the TK-Renilla luciferase control obtained from Promega. All assays were performed in triplicate. The depicted data is represented as ratios of normalized reporter activity with exogenous PTEN divided by the normalized reporter expression with the vector alone. These data show that the TRAIL reporter is well induced by PTEN alone whereas the other reporters are only modestly induced by PTEN.

The TRAIL reporter can be used to asses the level of Pi3K flux in different cell lines. We have carefully characterized the ability of the TRAIL reporter to be used as a read-out of Pi3K pathway activity and have prepared this work for publication. Much to our chagrin, the TRAIL reporter system, the best one that we identified for a potential PTEN screen tool, did not have a broad enough dynamic range for performing an RNAi screen. For example, RNAi to AKT, a key pathway component, only led to a 1.7 fold induction in reporter activity.

We have shifted our screen set-up from using a reporter based screen to using an inducible form of PTEN in suppressor/enhancer screens. I am in the process of building inducible PTEN cell lines. I have tried to do this in LNCaP, PC3 and DU145 backgrounds. To date, I have only been successful at obtaining a PTEN-inducible PC3 cell line. This cell line shows induction of PTEN that is lethal to cells. This cell line is currently being optimized and validated for RNAi screening and shows tremendous promise. We expect to begin screening this spring.

Key Research Accomplishments

1. PTEN mutant analysis
    - Constructed expression constructs for 23 PTEN mutants
    - Set-up functional assays to test the PTEN mutants
    - Directly tested the main hypothesis of this task, which was to determine whether PTEN functions are separable; based on my work we found that subsets of them are.

2. Screen set-up
    - Set-up a reporter assay for PTEN signal transduction
    - Determined that the reporter system was not suitable for large-scale screening
    - Wrote a paper about this reporter system
    - Designed a new set-up for large scale screening using an inducible form of PTEN

Reportable Outcomes:

- Most of the studied PTEN mutants fail to perform all of its tested functions.
- Five mutants perform only subsets of PTEN functions; this is an extremely important finding for several reasons. One, certain functions of PTEN are separable. Two, at least two functions ascribed to PTEN appear to be important for preventing cancer as some mutants found in tumors perform one set of PTEN functions whereas another subset of mutants performs other PTEN functions. Three, these mutants provide mechanistic insights into the ability of PTEN to perform a diverse set of cellular roles. These mutants indeed map to particular regions within PTEN that appear to direct it to different cellular roles. We are investigating these mechanisms now. This work should be published soon.
- I designed an extremely useful reporter set-up for the assessment of the level of PTEN signaling in a cell-based system. Although this reporter was not suitable for large scale screening, we are using it for drug discovery and as a barometer of PTEN signaling in general. We wrote a paper about this work, which will be published this year.
- We have identified an inducible system for performing a large-scale PTEN screen.
- Publications during reporting period:
    1. Janusz Puc, Megan Keniry, Hong Shen Li, Tej Pandita, Lorenzo Memeo, Mahesh Mansukhani, Vundavalli V. V. S. Murty, Sarah E. M. Meek, Helen Piwnica-Worms, Hanina Hibshoosh, and Ramon Parsons. 2005. "Lack of PTEN inhibits CHK1 and initiates genetic instability," Cancer Cell: 7:193-204.
    2. Megan Keniry, Janusz Puc, and Ramon Parsons, "Evaluating endogenous Pi3K signaling using a TRAIL luciferase reporter," *In Preparation.*
- Poster Presentation:
    AACR Annual Meeting in Anaheim, California, April 2005
    Title: Functional Analysis of PTEN mutants

Conclusions

The work detailed in this report was aimed at utilizing genetic analyses to investigate the mechanisms by which the PTEN tumor suppressor prevents cancer. First, I studied mostly tumor derived PTEN mutants for the ability to perform a diverse set of its functions. We found that most of the mutants failed to perform any of its known functions, suggesting that these cellular roles are indeed important for the inhibition of cancer. In addition to this, we found five mutants that performed distinct subsets of its functions. This is an extremely important finding as it suggests that numerous functions of PTEN are separable and that they are relevant for cancer progression. Furthermore, the paradigm for the role of PTEN as a tumor suppressor will shift as a result of our work as it appears to be not solely derived from its ability to regulate AKT. We found numerous mutants that regulate AKT as well as wild-type PTEN, but fail to perform other PTEN functions; these mutants were

identified in tumors. We will more rigorously test these results, but they have strong implications for the precise roles of PTEN as a tumor suppressor. The second task for this project is to identify novel components involved in PTEN signaling by performing a screen. Toward this goal, we devised a reporter system to measure PTEN signaling and have written a publication. This reporter will be a tool that has the potential to identify drugs as well as novel components that act on the PTEN pathway. This will aid in the identification of novel chemotherapies for prostate cancer. Although, the TRAIL reporter system is useful to assess PTEN signaling, it is not dynamic enough for large scale screening. We have developed an inducible system that dies upon PTEN induction for large scale screening.

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